ASSAY OF HISTIDINE DECARBOXYLASE INHIBITORS IN HUMAN PLASMA, RAT PLASMA AND RAT GASTRIC TISSUE

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INTRODUCTION

Histamine has been postulated as an important factor in a number of physiological processes (10), such as fetal growth, wound healing, the synthesis of granuolcytes in bone marrow, mediation of microcirculation (27) and hormone action (31), and gastric acid secretion (15). It is also thought th play a role in a number of pathological states (3), such as anaphalaxis, myelogenous leukemia, mastocytosis (16), Zollinger-Ellison syndrome (15), thyrotoxicosis, periarteritis nodosa, asthma, hay fever (25), and peptic ulcer (4). As an increased level of histamine has been noted in many of these situations, it is understandable that interest would be focused on the enzyme responsible for synthesis of histamine, histidine decarboxylase, and on any inhibitors of this enzyme. In this thesis the present knowledge concerning histidine decarboxylase, methods for assay of the enzyme, and agents that inhibit it will be surveyed briefly. Also, as background for the studies performed, certain physiological aspects of histamine with emphasis on its proposed role in gastric acid secretion will be discussed. The experimental section of this thesis is concerned

with the development of a technique for assay of histidine decarboxylase inhibitors in plasma. The drugs studied were the hydrazino analog of histidine (MK-785), and 4-bromo-3-hydroxybenzyloxyamine (brocresine).

The first approach to the problem that was attempted was based on a report by Krauss, et al.(13). The authors, choosing patients with disease states in which leukocytes had higher than normal levels of histidine decarboxylase, detected decreased levels of both histamine and histidine decarboxylase activity in the granulocytes from patients with chronic myelogenous leukemia after administration of brocresine. An attempt was made to use the histamine level in granulocytes of normal volunteers as an index of histidine decarboxylase inhibition in the plasma. Unfortunately, the amount of blood needed to detect histamine in the white cell fraction was impractical, and consistent levels could not be detected with 100 cc of blood, so this approach was abandoned.

The next approach to the problem proved successful. A radioisotopic assay for histidine decarboxylase activity previously described by Levine and Watts (19) was adapted so as to accurately assay histidine decarboxylase inhibition in as little as 5 cc of human blood. The experimental section of this thesis describes this assay as well as the results obtained by application of this assay in both man and rat. In the human studies known concentrations of both MK-785 and brocresine in pooled human plasma were assayed in vitro and curves were constructed from which values from subsequent in vivo studies could be expressed as actual concentrations of the drug. Next, the plasma levels of both

MK-785 and brocresine were determined at various time intervals after various peroral doses of the two drugs. From this, dose-response curves were constructed showing actual plasma concentration of the drugs with respect to time after administration. In the rat, intraperitoneal doses of MK-785 and brocresine were correlated both with the plasma level of the drugs and the level of histidine decarboxylase inhibition in stomach tissue.

The data from these studies allow a number of conclusions and correlations to be made. The studies done in humans provide information concerning rate of absorbtion and clearance of MK-785 and brocresine after various oral doses, and plasma concentration levels reached with these oral doses. The studies done in rats provide data concerning inhibition of histidine decarboxylase in tissue and correlation of this inhibition with concurrent plasma levels of the drugs. Presuming that the relationships between plasma levels and enzyme inhibition exist in man as were demonstrated in rats, the dose-response curves which resulted from the human studies are useful in the development of clinical dosage schedules.

REVIEW

Histamine is synthesized in mammalian tissue by decarboxylation of its precursor amino acid, histidine. This is accomplished by a specific histidine decarboxylase. Although histidine is also decarboxylated *in vitro* by a non-specific aromatic L-amino acid decarboxylase, it is generally concluded that only the specific histidine decarboxylase functions *in vivo* to synthesize histamine (17). This conclusion

is based in part on the low affinity of the non-specific decarboxylase for histidine (22). Furthermore, specific inhibitors of the specific histidine decarboxylase, such as α-methyl histidine (10), decrease histamine levels in tissue and urine of rats, whereas administration of a selective inhibitor of the non-specific decarboxylase, α-methyl dopa (10,17), has no effect on either urinary or tissue histamine levles. The enzyme, histidine decarboxylase, is specific for the substrate histidine (5-8), requires pyridoxal-5'-phosphate as a cofactor (5,6), has a pH optimum which varies inversely with substrate concentration (from pH of 6.2 for 5×10^{-2} M histidine to pH of 7.2 with 5×10^{-6} M substrate) (5,6,7), and a K_m which varies inversely with pH (pK_m is 1.5 at pH of 4.0, pK_m is 5.5 at pH of 9.0) (1,7). On the basis of these data, although the enzyme is more active at acid pH values, it is thought to interact only with the anionic form of histidine as substrate, an ionic species which is more abundant in alkaline media.

The structural formulas of the two histidine decarboxylase inhibitors used in the following studies, MK-785 and brocresine, are shown in Figure 1. MK-785, the hydrazine analog of the substrate histidine, functions as a reversible (19) competitive inhibitor by interacting with the cofactor pyridoxal phosphate. In vivo, MK-785 is a more potent inhibitor of histidine decarboxylase than of aromatic L-amino acid decarboxylase, whereas brocresine strongly inhibits both enzymes. By contrast brocresine (4-bromo-3-hydroxybenzyloxyamine) appears to function as a competitive irreversible (19) inhibitor by forming an oxime by reaction with the cofactor and then complexing

with the enzyme (21); this oxime has been shown to be a non-competitive inhibitor of the reaction (21).

Several procedures are available to assay histidine decarboxylase activity *in vitro*; these are compared by Maudsley, *et al.*(23). Selection of an assay procedure is a controversial issue; some investigators do not accept results of an assay other than their own. One of the controversial points is the preparation of tissue. Some assays use intact cells, obtaining results which differ from those studies in which cell-free homogenates are used (10). Furthermore, some assays use a nitrogen atmosphere, which may or may not affect enzyme activity (1).

In assays where the tissue is homogenized, the supernatant is at times purified by gel filtration, ammonium sulfate precipitation or dialysis to remove endogenous activators, inhibitors and cofactors. It is easy to see that variation in these techniques might quite easily lead to variable results. The means of detection of histidine decarboxylase activity is another variable facet of these assays. Nonisotopic methods are usually too insensitive to detect the amount of enzyme present in most tissues studied. Of the two isotopic methods in wide use, ring labeled C^{14} histidine as substrate, in which the product is a C^{14} histamine derivative, is the more highly specific but more time consuming method. A simpler method, and the basis of the assay used in this study, uses C^{14} histidine labeled in the carboxyl carbon; enzyme activity is measured by the trapping of the evolved $C^{14}O_2$ in hydroxide of hyamine for subsequent scintillation counting.

The problems in this method primarily involve high blank values due to nonenzymatic decarboxylation in the reaction mixture. Since this method employs a stomach homogenate in the reaction mixture which is not purified further by differential precipitation or gel filtration, it may contain substances which either inhibit or enhance the enzyme reaction. Although substantial time has been devoted to the development of a valid blank, this problem has not yet been solved, and contributes to the variability in parts of the present study. Certain steps, such as heating the substrate prior to use, have given reasonably valid blank values. In summary, the variation in tissue preparation as well as in the product detection method in the various assays of histidine decarboxylase is a controversial area which is not yet resolved. The method used in these studies is not as highly specific as those methods based on C^{14} ring labeled histidine; however it is specific, highly sensitive and rapid, and is adaptable to a number of clinical and laboratory situations.

As previously mentioned, histamine is thought to be a significant factor in a variety of physiological and pathological states (8,10). High histidine decarboxylase activity has been found in relation to many of these states, such as in fetal tissues, granulation tissue, some tumors and the gastric mucosa of many animals and man. The relationships of histamine and histidine decarboxylase activity in the gastric mucosa to gastric acid secretion in the rat have been studied in detail by several investigators. The rat gastric mucosa contains a large rapidly turning over pool of histamine (12,30) which

is released by a number of agents (9,29) which are known to stimulate gastric acid secretion. Soon after histamine and acid release, there is an increase in histidine decarboxylase activity in the gastric mucosa (10-12,26,30). The fact that the rat stomach contains a rapidly turning over pool of histamine and that histamine appears to be a mediator of gastric acid secretion in this aminal (2) makes this an opportune area for the study of histidine decarboxylase inhibitors. Treatment of rats with either MK-785 or brocresine results in decreased acid production both in basal states and following gastrin stimulation (14), as well as a significant decrease in stress ulcer formation in response to cold and restraint (18,28). In these studies gastric acid secretion occurred in response to administered histamine, indicating functioning acid secreting cells and not merely nonspecific destruction of cellular function by the inhibitors (18).

The above finding tend to strongly implicate histamine as a mediator in gastric acid secretion in the rat (2). Extrapolation of this concept to primates and man was a dubious jump due to the inability to clearly demonstrate specific histidine decarboxylase in the gastric mucosa of species other than the rat (11). Recently, however, significant specific histidine decarboxylase activity has been domonstrated in the gastric mucosa of man as well as other primates (24). Furthermore, clinical trials using brocresine in patients with Zollinger-Ellison syndrome, a disease characterized by gastric hypersecretion secondary to a gastrin-like substance elaborated by a non-beta cell tumor of the pancreas, demonstrated a decrease in urinary histamine excretion and

and symptomatic relief while on histidine decarboxylase inhibitors (15). These recent findings point toward histamine as a mediator of gastric acid secretion in man as well as the rat. Availability of detailed investigations on rat stomach suggested this organ as the system in which to study the correlation of plasma inhibitor levels with tissue histidine decarboxylase inhibition.

EXPERIMENTAL

Although several methods are available to assay histidine decarboxylase activity *in vitro*, at the onset of these studies there were no methods available to assay plasma levels of histidine decarboxylase inhibitors or to correlate drug dosage with plasma level or tissue activity. As clinical trials (16,25,26) had already begun with two histidine decarboxylase inhibitors, MK-785 and brocresine, it appeared important to develop a rapid and sensitive assay which could be applied to any inhibitor of histidine decarboxylase, which required small amounts of blood in order to allow detailed dose-response curves to be constructed, and which could be used easily in clinical situations. Therefore a rapid assay was developed based upon estimation of the power of plasma to inhibit histidine decarboxylase activity after administration of a drug. This approach to the assay of drug levels was chosen because some histidine decarboxylase inhibitors, such as brocresine, are so potent that concentrations as low as 0.05 μM in plasma produce significant inhibition, and compounds at this concentration would probably defy detection by ordinary chemical means. However, a

method based upon histidine decarboxylase inhibition should be generally applicable to assay of plasma levels of any histidine decarboxylase inhibitor.

This method was applied to the study of plasma levels of MK-785 and brocresine after peroral administration of single doses to normal subjects, and to the study of plasma levels of rats after single intraperitoneal doses of either of the two inhibitors. The assay was modified to allow estimation of histidine decarboxylase activity in rat stomach tissue after single intraperitoneal doses of MK-785 or brocresine. Rat studies were performed to determine the correlation, if any, between plasma levels and tissue activity of the two histidine decarboxylase inhibitors. Dose-response curves in man permitted comparison between the two systems and allowed the relationship between plasma drug levels and tissue activity observed to be tentatively extrapolated to man.

MATERIALS AND METHODS

The two drugs used throughout this study are D-2-hydrazine-3-4(5)-imidazole proprionic acid (MK-785) supplied as a powder for use in the rat or as 50 mg. capsules for human use by Merck Institute for Therapeutic Research, and 4-bromo-3-hydroxybenzyloxyamine (brocresine), supplied as a powder or in 50 mg. and 200 mg. capsules by Lederle Laboratories.

Studies in man. Histidine decarboxylase was prepared from whole fetal rats (19 to 20 days gestation) by a modification (19) of the

method of Håkanson (6) in which homogenization of the fetal rats in pH 5.5 sodium acetate buffer was followed by differential precipitation of the crude mixture. Further purification was effected by dialysis. The enzyme preparation was stored at -20° C. until used for assay. Assay of histidine decarboxylase inhibition was performed by a modification of a previously described method (19). This method is based upon determination of the power of plasma from subjects who have ingested either MK-785 or brocresine to inhibit the activity of a preparation of histidine decarboxylase in vitro. The method employs incubation of the enzyme with C^{14} histidine (labeled in the carboxyl carbon) in the presence of excess co-factor, and trapping the evolved $C^{14}O_2$ in hyamine hydroxide for assay in a Packard "Tri-Carb" scintillation spectrometer. The incubation vials contained, in addition to excess co-factor, C^{14} histidine, and histidine decarboxylase, 0.8 cc human plasma, sodium phosphate buffer in order to maintain the incubation mixture at a pH of 6.8, and streptomycin sulfate, added to supress the growth of any bacteria which may decarboxylate histidine. In these experiments incubations were done in final volumes of 2.0 ml. Blanks consisted of complete incubation mixtures to which brocresine was added to a final comcentration of 0.1 μM and 0.8 cc water was substituted for human plasma.

Plasma from some subjects who had received no drug was found to produce small degrees of histidine decarboxylase inhibition; this inhibition was generally small (0-10%) but in some cases was greater. In these experiments this inhibition was equated to zero by designating

the control (pretreatment) value for any subject as zero percent inhibition.

The subjects of these studies were 27 normal volunteers, ages 20 to 46, of which 14 were white females, two, negro females, and eleven, white males. Venous blood was aspirated into 10 ml. heparinized Vacutainer (r) tubes (3200 KA) before and at various times after ingestion of the drug. Plasma was separated by centrifugation and stored at -20° C. until assay.

Studies in the rat. Female CD rats (derived origionally from Sprague-Dawley stock) were purchased from Charles River Laboratories, North Wilmington, Massachusetts. At the time of study they weighed between 140 and 180 g. In the studies determining the plasma levels of the two drugs with respect to time post injection, approximately ten rats were used for each point. Each rat was given a dose of 150 mg./kg. of either MK-785 or brocresine intraperitoneally. The rats were sacrificed either at five, 15, or 30 minutes or at one, two, four, eight or 16 hours after injection. Blood was obtained by cardiac puncture and aspiration into an heparinized syringe and 0.8 ml. plasma was assayed for histidine decarboxylase inhibitory activity by the method described in the human study.

In studies correlating inhibition of histidine decarboxylase activity in stomach tissue with elapsed time after injection of the drug, ten rats were used for each point: seven experimental and three controls. A similar protocol was followed with respect to the dose and the time intervals used. Controls were injected with normal saline. All rats were injected and left in their cages in the Animal Care

Division with free access to food and water until sacfificed. Stomachs were trimmed, washed and blotted dry prior to assay for histidine decarboxylase activity as described by Levine and Watts (19). The method differs from the assay described previously in human studies in that 0.8 cc stomach homogenate was used in place of human plasma, while purified fetal rat histidine decarboxylase was not added to the incubation mixture. Protein in the stomach tissue was measured by a modification of the phenol reagent method (26). Histidine decarboxylase activity was expressed as counts/min./mg. protein.

RESULTS

Studies in man. In preliminary experiments, MK-785 and brocresine were dissolved in pooled human plasma; serial dilutions were made by addition of plasma. Inhibition of histidine decarboxylase activity by drugs dissolved in plasma *in vitro* is illustrated in Figures 2 and 3. For MK-785, histidine decarboxylase inhibition was a linear logarthmic function of drug concentration in plasma over a range of 0.1 to 100 μM. For brocresine the range of concentration that could be studied reliably was smaller, ranging from 0.1 to 1.0 μM. These curves were used to standardize subsequent studies on plasma levles of histidine decarboxylase inhibitors *in vivo*.

Figures 4 and 5 illustrate plasma levels of MK-785 and brocresine resulting from oral administration of single doses. These figures illustrate that the plasma levels obtained with oral administration of these drugs varies substantially between subjects. This variation was more striking with MK-785 than with brocresine.

Figures 6 and 7 illustrate plasma levels of MK-785 and brocresine after oral administration of various doses. Again differences between subjects were noted. These studies revealed some major differences between the two inhibitors. Peak plasma levels of MK-785 were much higher than those obtained with equal doses of brocresine. For example, after an oral dose of 50 mg. of MK-785, plasma levels reached a peak of 0.5 to 0.6 μM while double that dose of brocresine produced plasma levels that were barely detectable. Peak levels in plasma were reached much earlier with brocresine (30 to 45 minutes) than with MK-785 (two to four hours). Also, it was apparent that brocresine disappeared from plasma much more rapidly than did MK-785.

Studies in the rat. Data from determination of plasma levels of both MK-785 and brocresine at various times after parenteral administration are shown in Figures 8 and 9. Results are plotted semi-logarithmically as a ratio of the average control value and the average experimental value; high numbers indicate greater concentration of histidine decarboxylase inhibitors in the plasma. MK-785, as shown in Figure 8, reaches peak levels in one hour, and maintains fairly high levels for our hours. Brocresine, however, reaches its peak plasma level in about 15 minutes and is essentially cleared from the blood within one hour.

The relationship of histidine decarboxylase activity in stomach tissue to time elapsed after parenteral injection of either MK-785 or brocresine is shown in Figures 10 and 11. Individual values, originally expressed as counts/min./mg. protein, were converted to percent inhibition. Blank values in this study were not subtracted

from experimental values.* While this leads to a less dramatic curve of histidine decarboxylase inhibition in stomach tissue, it indicates the time course of drug activity in the tissue and also points out some of the problems previously mentioned concerning the assay when applied to tissue having low enzyme activity. In Figure 10 it can be seen that histidine decarboxylase in the stomach is highly inhibited between 15 minutes and four hours after injuction of MK-785, but enzyme activity returns to normal after ten hours. In contrast, brocresine (Figure 11) inhibits gastric histidine decarboxylase activity maximally between 30 minutes and two hours, maintaining only minimal levels of enzyme inhibition after four hours.

DISCUSSION

The experiments described above indicate the feasability of assaying histidine decarboxylase inhibitor levels in plasma by determining enzyme inhibiting potency of plasma after drug admin-

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* Frequently blank values were higher than experimental points supposedly representing high histidine decarboxylase inhibition. This was probably due to various substances in stomach homogenates which either enhanced or inhibited the decarboxylation reaction but were not represented in a blank which contained only water and buffered solution as substitute for the homogenates.

istration. This constitutes the first report of a method for assay of these agents in plasma. Reasons for development of this technique and the particular advantage of its general applicability have been commented on earlier. However, the assay is not specific for any given reagent but will reflect the presence of all chemical or metabolic derivatives that inhibit histidine decarboxylase.

Comparison of drug plasma curves of either MK-785 or brocresine in man and in the rat will show that they behave similarily in both species. In man, MK-785 maintains high plasma levels for about eight hours with 400 mg. doses; with 200 mg. doses, plasma levels were significantly decreased after eight hours, closely resembling effects in the rat. Brocresine in man maintains peak levels only for 30 minutes to one hour, with significant decrease by two hours. The concentration curve of the drug in rat plasma, after parenteral administration shows even more rapid clearance, but in general is similar. Comparison of the plasma curves with tissue activity in the rat seems to demonstrate rapid equlibrium between plasma and tissue for the two drugs, with histidine decarboxylase inhibition demonstrable for only one to three hours after the drug is cleared for the plasma. This is consistant, in the case of a proposed irreversible inhibitor such as brocresine, with enzyme half lives of 100 (12) to 120 (30) minutes in the rat gastric mucosa. The data suggest that brocresine is absorbed rapidly and equilibrates rapidly with gastric tissue; the length of demonstrable histidine decarboxylase inhibition appears to be a function of resynthesis of the enzyme after irreversible inhibition. Brocresine itself is cleared from rat plasma after two hours and is probably also absent

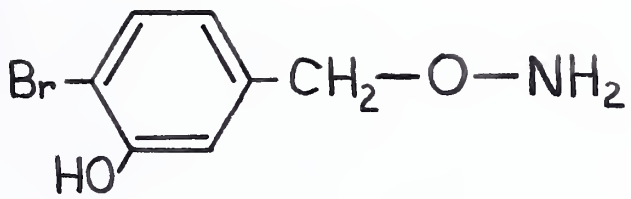
from gastric mucosa equally soon. Studies of MK-785, thought to be a reversible inhibitor, were less precise and may merely reflect clearance of the drug from plasma and tissues after several hours. Recovery from this type of inhibition should not be dependent on synthesis of new enzyme.

That brocresine is cleared from the plasma in three hours in man does not necessarily indicate that it has been cleared from the body. But the rat studies suggest that tissue levels of these histidine decarboxylase inhibitors parallels plasma levels with a residual inhibitory phase of a few hours, and perhaps a similar relationship holds in man. Until further data are available, the assumption that maximum plasma levels may parallel maximum enzyme inhibition in some tissues may serve as a guide in construction of dosage schedules. Before this new this new technique became available, several studies using brocresine were in progress in both normal subjects and in patients with various diseases. Doses used in these studies were either 200 or 400 mg. every eight hours. The present studies indicate that, although the doses were sufficiently large, administration was too imfrequent to maintain effective plasma concentrations of the drug. When the frequency of administration was changed to every four hours, clinical results were improved (20,25). Thus the data presented here should be of value in future clinical trials with MK-785 and brocresine, and the availability of this method to assay plasma levels of any histidine decarboxylase inhibitor should prove to be a useful adjunct to clinical trials of these drugs.

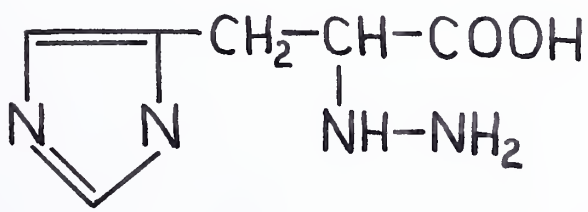
SUMMARY

A technique for the assay of plasma levels of histidine decarboxylase inhibitors in man has been described. The assay is based upon plasma inhibition of histidine decarboxylase activity in vitro after drug administration. This method was used to study plasma levels of MK-785 and brocresine after administration of various doses in both man and rat. A similar assay, described previously, was used to estimate histidine decarboxylase activity in rat gastric mucosa after inratperitoneal injection of either drug.

Correlations between plasma levels and gastric tissue enzyme inhibition were found, and tentative conclusions were made regarding similar relationships in man. Based on our results, alterations were made in dosage schedules in clinical trials using brocresine. The assay technique described should prove useful in future clinical trials with either MK-785 or brocresine, and should be generally applicable fo quantitative determination of any histidine decarboxylase inhibitor.



BROCRESINE



MK-785

Figure 1. Structural formulas of the two histidine decarboxylase inhibitors used in this study.

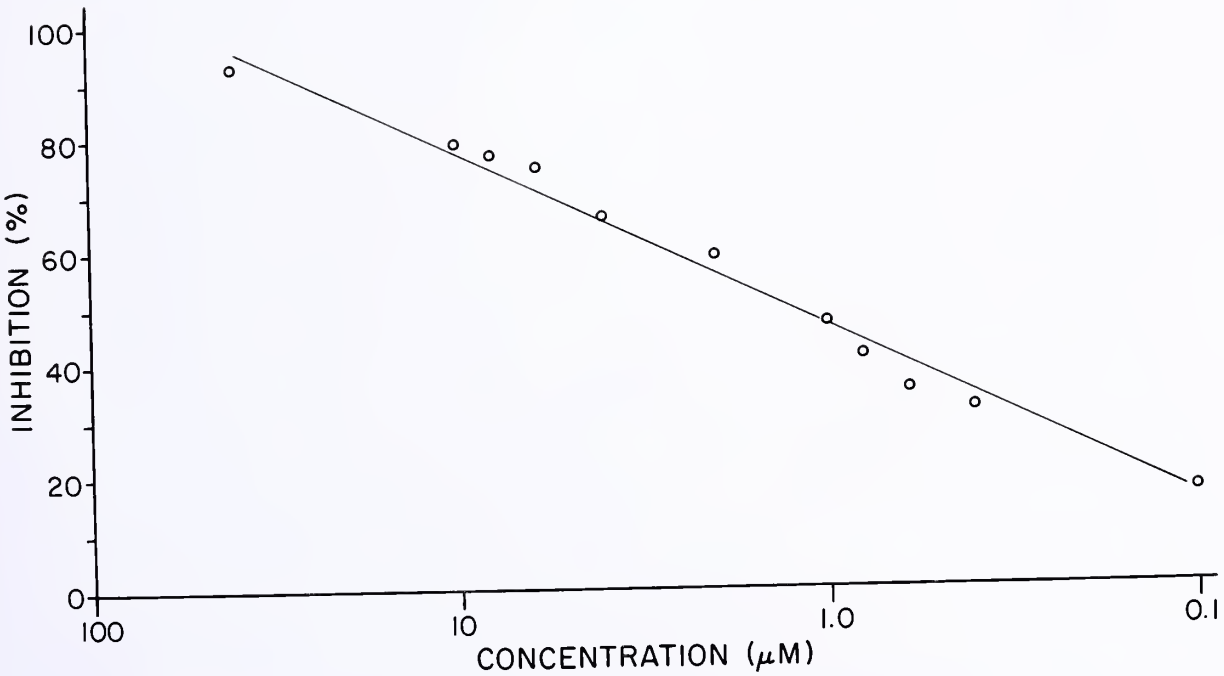


Figure 2. Inhibition of histidine decarboxylase activity by MK-785 dissolved in plasma in vitro. The concentration designated on the abcissa is the concentration of MK-785 in plasma, of which 0.8 ml. was added to the incubation mixture to a final volume of 2.0 ml.

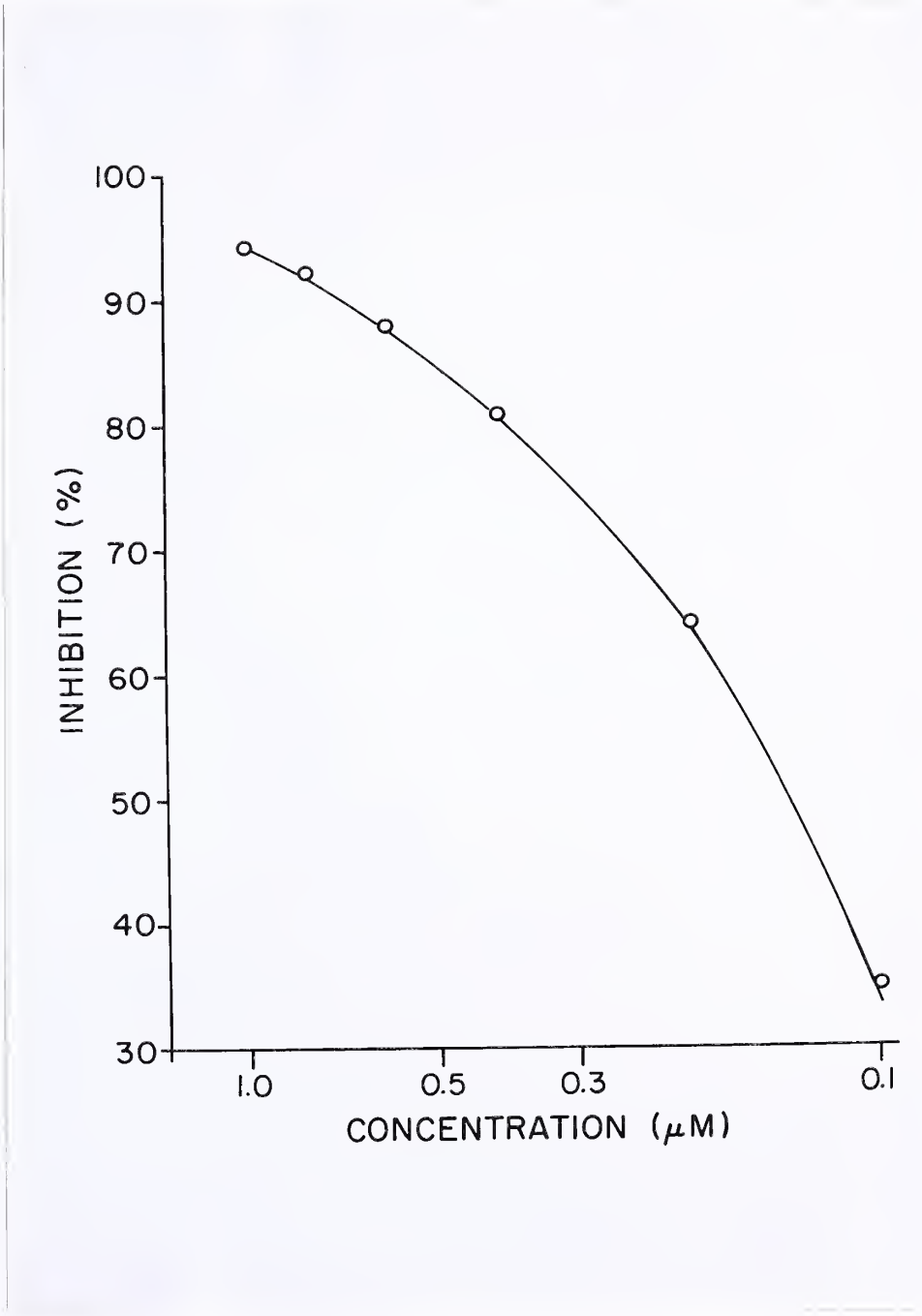


Figure 3. Inhibition of histidine decarboxylase activity by brocresine dissolved in plasma in vitro. The concentration designated on the abcissa is the concentration of brocresine in plasma, of which 0.8 ml. was added to the incubation mixture to a final volume of 2.0 ml.

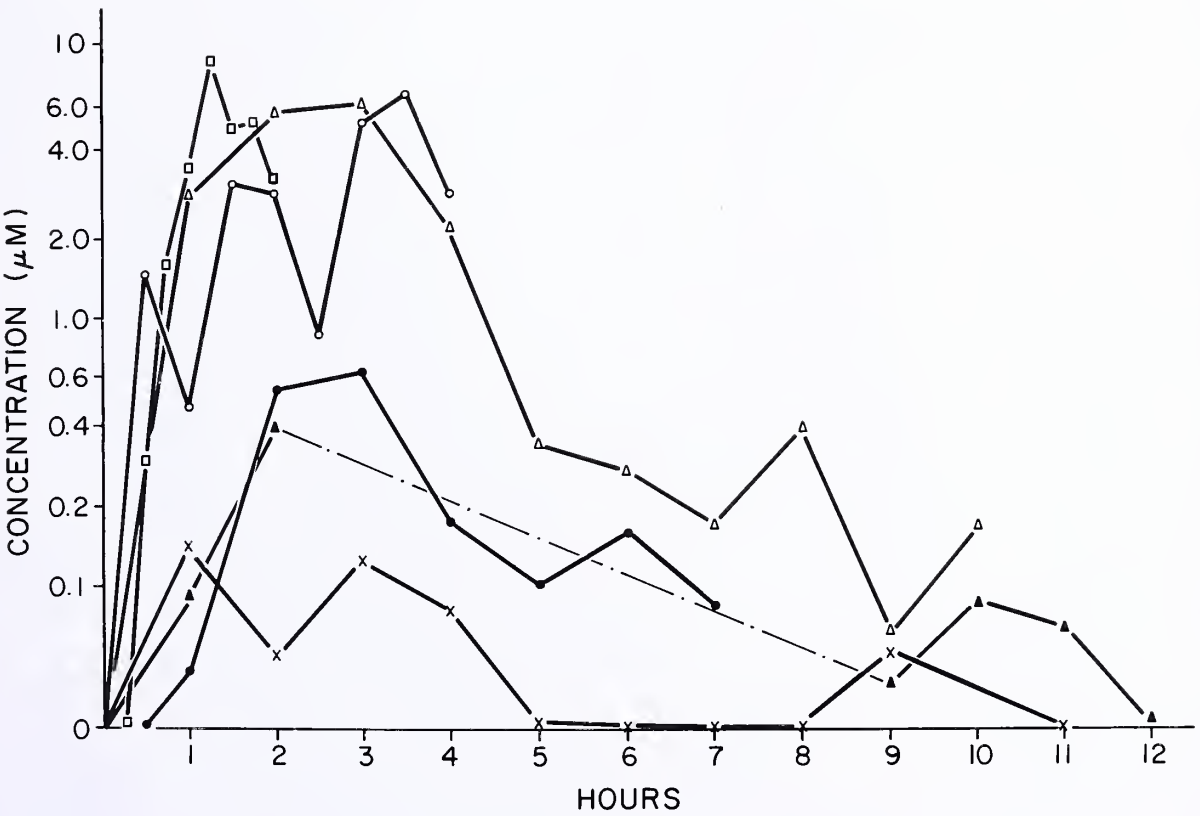


Figure 4. Concentrations of MK-785 in plasma of six individuals at various times after oral administration of a single dose of 200 mg. at zero time.

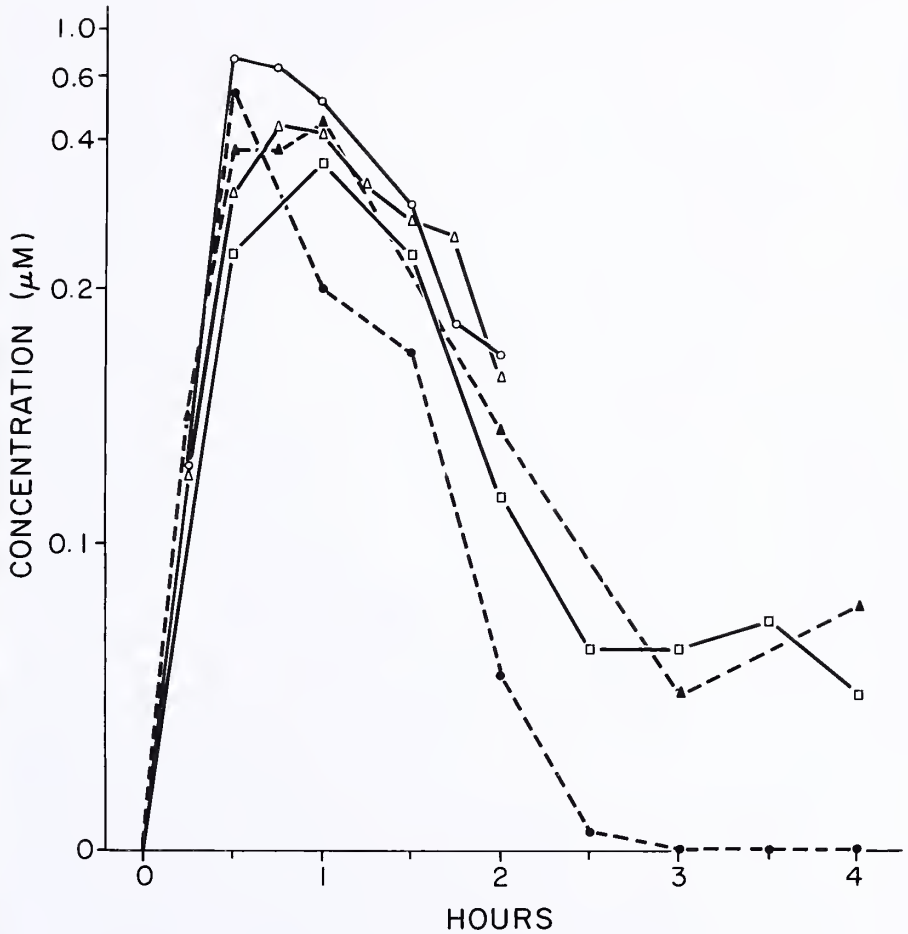


Figure 5. Concentrations of brocresine in plasma of five individuals at various times after oral administration of a single dose of 400 mg. at zero time.

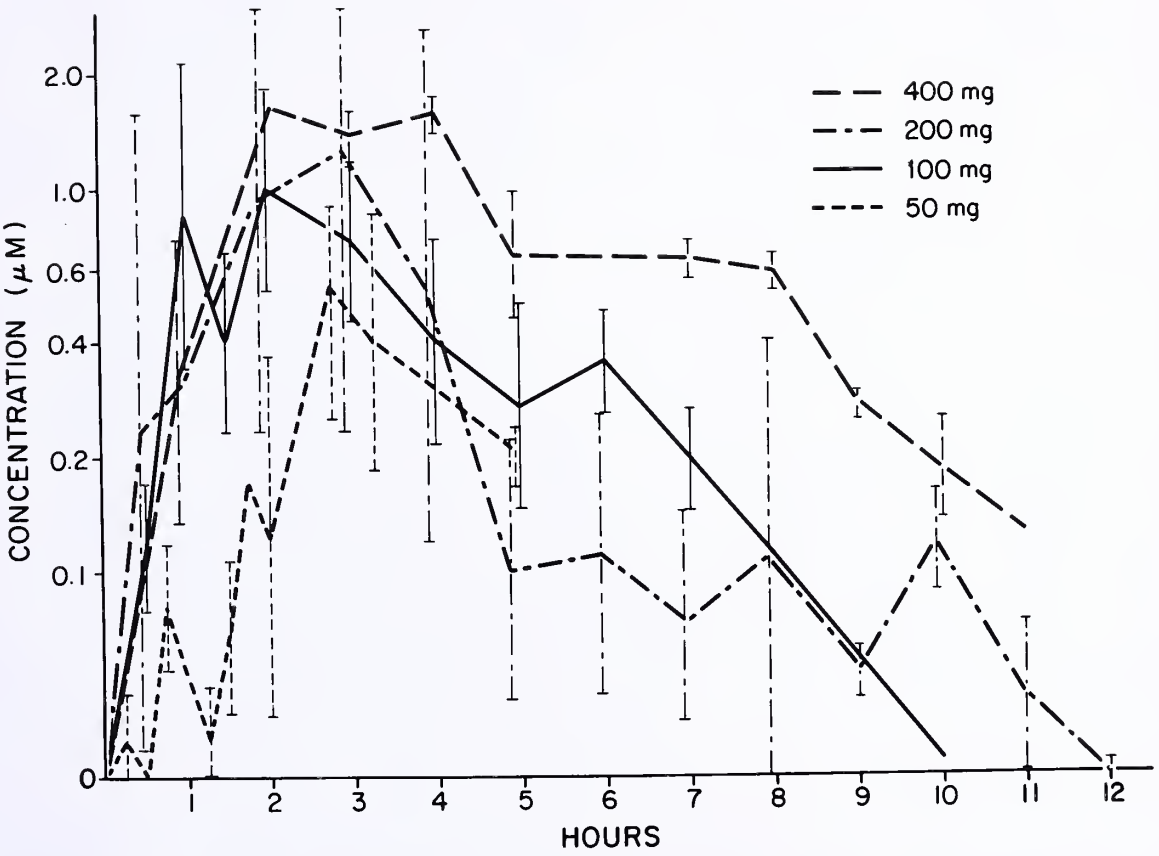


Figure 6. Plasma levels of MK-785 at various times after oral administration of various single doses. The data are plotted as mean concentrations in all subjects studied. At those points at which results are available from three or more individuals, there are vertical brackets indicating mean deviations (sum of deviations from the mean divided by number of observations).

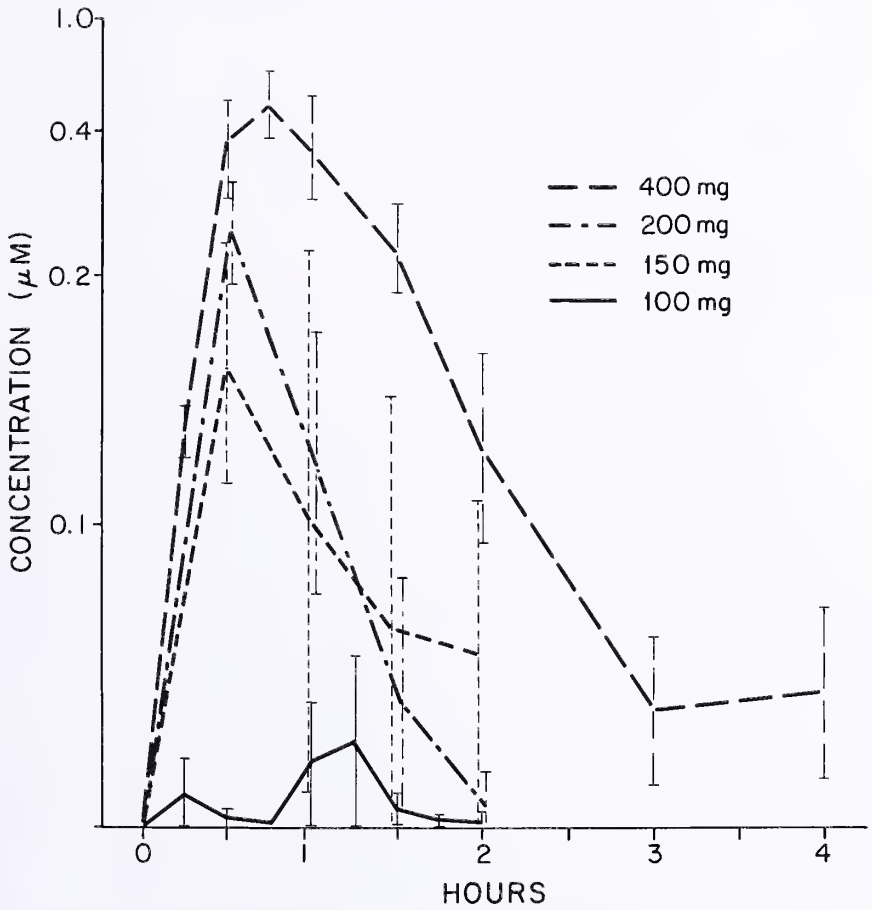


Figure 7. Plasma levels of brocresine at various times after oral administration of various single doses. The data are plotted as in Figure 6.

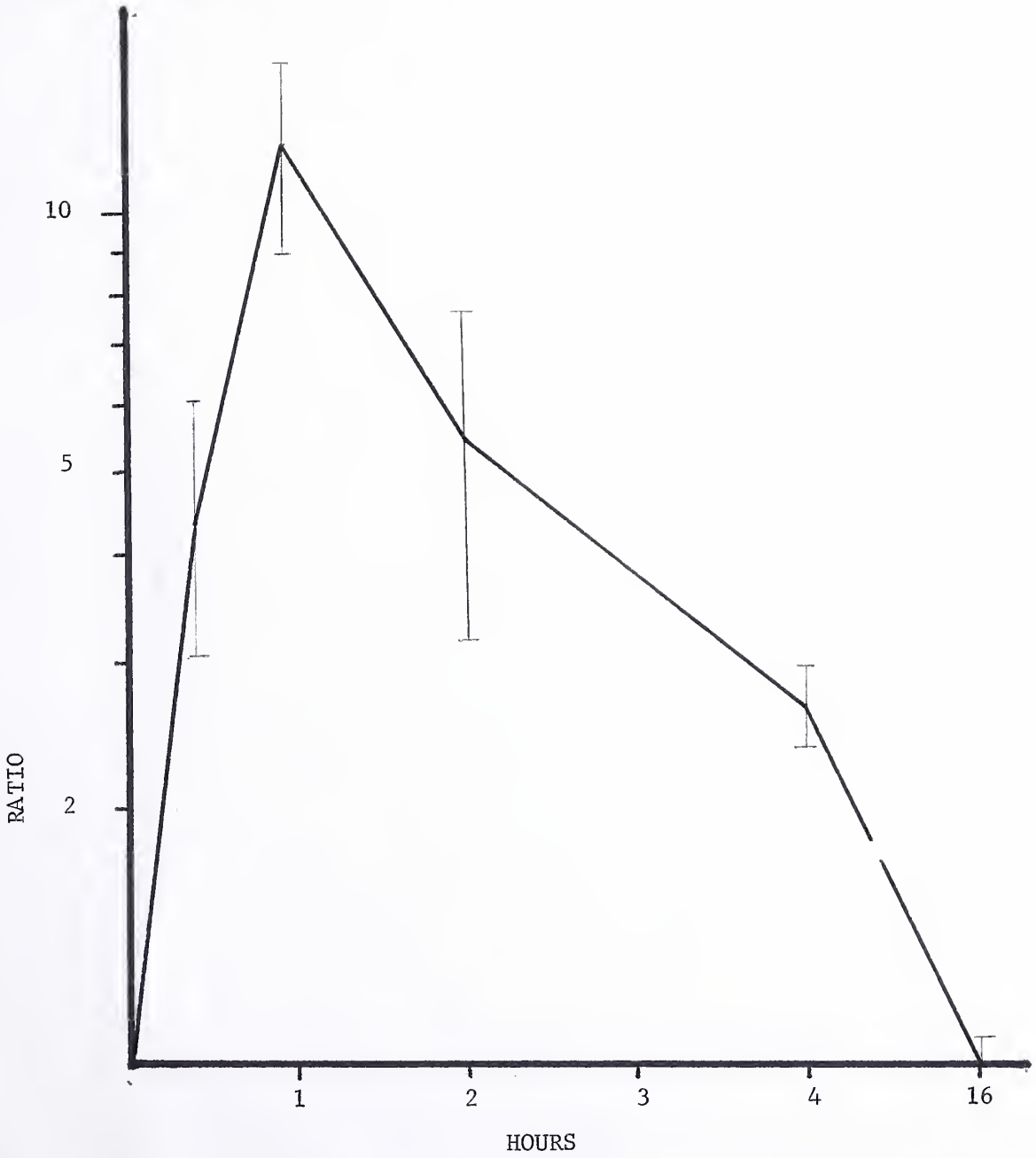


Figure 8. Plasma levels of MK-785 in the rat at various times after intraperitoneal administration of a single dose of 150 mg./kg. at zero time. Brackets indicate ± one standard deviation. The values on the abcissa indicate the ratio of the average control value divided by the average experimental value.

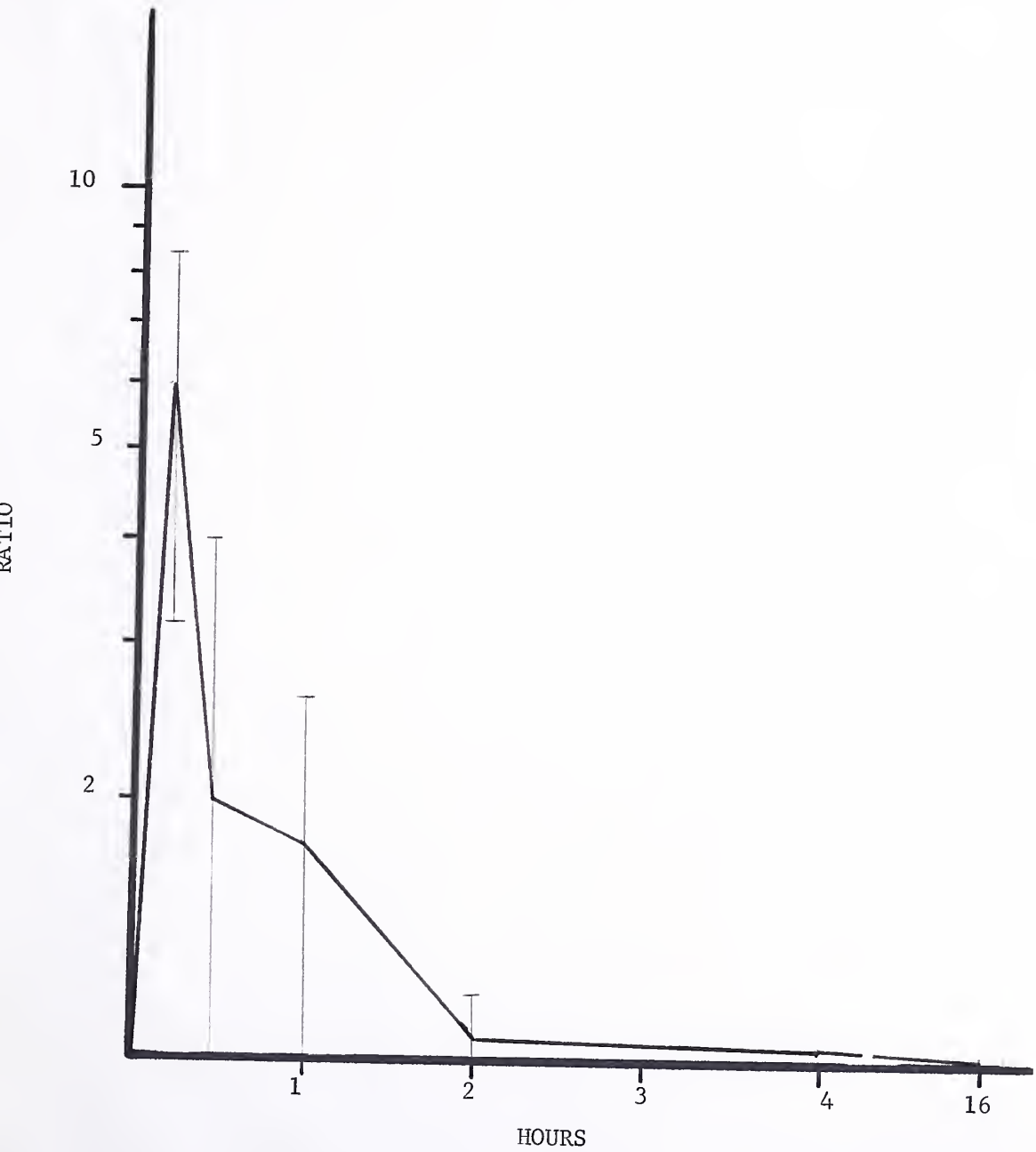


Figure 9. Plasma levels of brocresine in the rat at various times after intraperitoneal administration of a single dose at zero time. The dosage and data are plotted as in Figure 8.

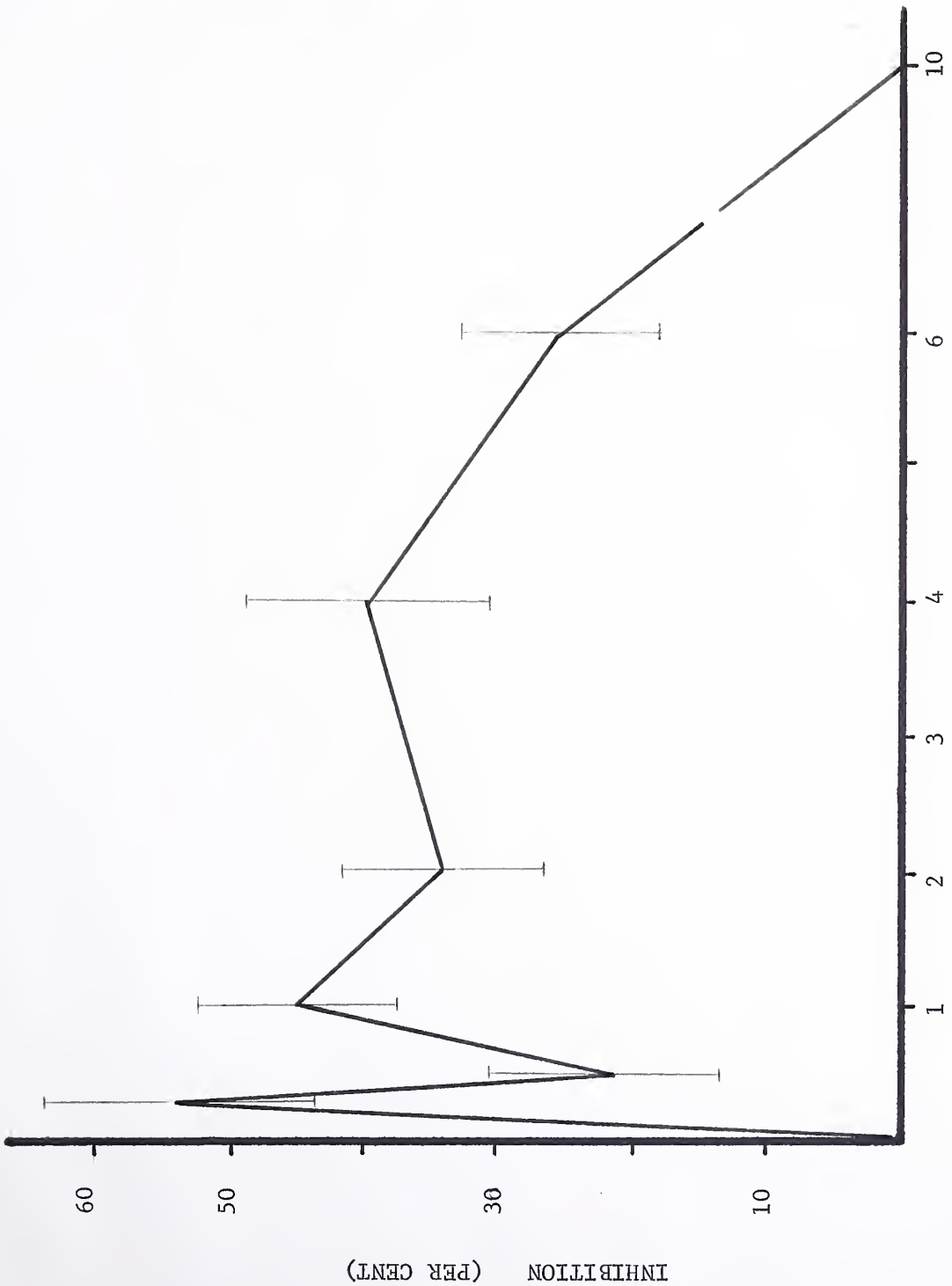


Figure 10. Inhibition of histidine decarboxylase in the rat gastric tissue at various times after intraperitoneal administration of a single dose of MK-785 at zero time. Dosage is as in Figures 8 and 9.

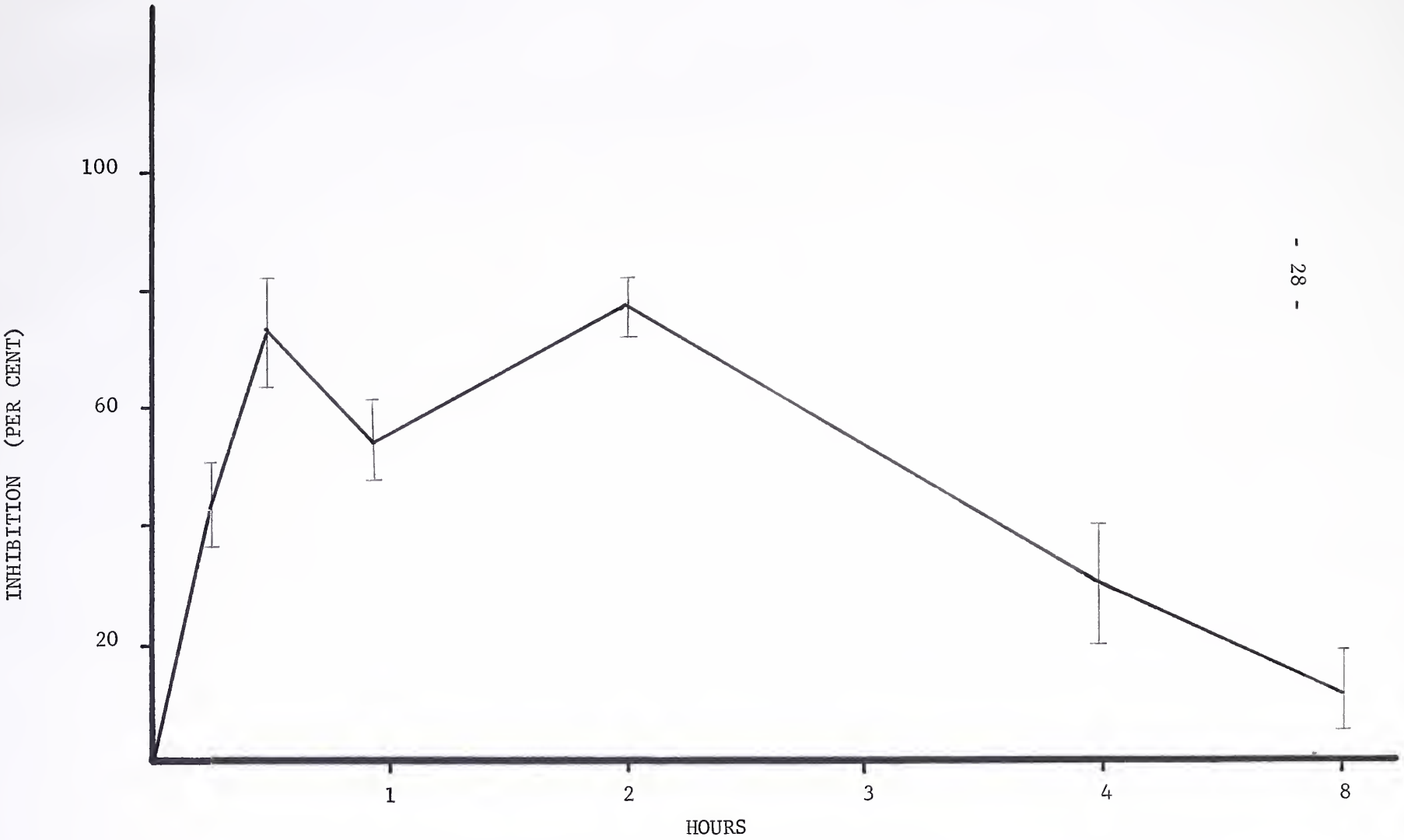


Figure 11. Inhibition of histidine decarboxylase in rat gastric tissue at various times after intraperitoneal administration of a single dose of brocresine at zero time. Dosage is as in Figures 8,9 and 10.

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